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RESEARCH PAPER

Activities of lipoxygenase and phenylalanine ammonia lyase in poplar leaves induced by insect herbivory and volatiles

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Abstract: A study was conducted to explore the defense response in woody plants after insect herbivory. The activities of two enzymes, lipoxygenase (LOX), a key enzyme of jasmonate (JA) pathway, and phenylalanine ammonia lyase (PAL), a rate-limiting enzyme of phenylpropanoid pathway, were measured in the leaves of one-year-old poplar (*Populus simonii* × *P. pyramidalis* 'Opera 8277') cuttings after *Clostera anachoreta* larvae attack. The results show that the increased activities of LOX and PAL were found not only in the leaves wounded by *C. anachoreta* larvae but also in their upper systemic leaves, indicating that JA and phenylpropanoid pathways were activated, and the defense response was stimulated systemically. The increase in LOX and PAL activities in neighboring intact poplar cuttings suggested that there exists the interplant communication between poplar plants mediated by the herbivore-induced volatiles. Methyl jasmonate (MeJA) was also proved to be an airborne signal to induce defense response in *P. simonii* × *P. pyramidalis* 'Opera 8277' cuttings.

Keywords: *Clostera anachoreta* larvae; lipoxygenase; methyl jasmonate; phenylalanine ammonia lyase; *Populus simonii* × *P. pyramidalis* 'Opera 8277'cuttings

Introduction

Plants use many strategies to defend against herbivore attack. In addition to physical barriers, such as thorns, trichomes and waxes, these defenses can include both secondary metabolites and proteins, which can act as toxins, antinutrients, or antifeedants (Constabel 1999). The rapid synthesis of proteinase inhibitors (PIs) that can decrease insect growth rates and increase mortality via inhibiting the digestive proteases of insects is one of the most important defense responses in plants (Ryan 1990; Major and Constabel 2008). Jasmonate (JA) pathway that is a well known signaling pathway in plants mediates inducible expressions of PIs (Farmer et al. 1992). The JA-synthesis mutant *def-1* is deficient in induced accumulation of JA and PIs, so the two-spotted spider mite feeding and fecundity on *def-1* plants were significantly greater than on wild-type plants (Li et al. 2002a). In addition, JA pathway also contributes to inducible expressions of many other defense genes in plants (Creelman and mullet 1995; Titarenko et al. 1997; Wang et al. 2008). Lipoxygenase (LOX) that can catalyze the oxygenation of fatty acids to their hydroperoxy derivatives leading to the production of 13-hydroperoxy-octadecatrienoic acid during the biosynthesis of JA is the key enzyme of JA pathway (Feussner and Wasternack 2002; Turner et al. 2002). High expression of LOX has been found to be induced by insect herbivory in *Nicotiana attenuata* (Pandey et al. 2008). However, the role of LOX in woody plants in response to insect herbivory is little known.

The secondary plant metabolites also play a key role in plant defense against herbivore attack (Constabel 1999). Phenylpropanoid pathway is an important pathway to produce secondary plant metabolites (Dixon and Paiva 1995). Products of stress-induced phenylpropanoid pathway are commonly lignin or lignin-like polymers that are important for cell wall reinforcement and may include phenolics (Dixon and Paiva 1995).

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Phenylalanine ammonia lyase (PAL), as a key enzyme, catalyzes the deamination of phenylalanine to cinnamic acid, the entry and key regulatory step into the phenylpropanoid pathway (Ritter and Schulz 2004). PAL has been found to be induced by wounding and insect herbivory, pathogen infection and abiotic stresses (Hahlbrock and Scheel 1989; Dixon and Paiva 1995), but the role of PAL is large unknown in defense response of poplar plants to insect herbivory.

In addition to direct defense, such as productions of defensive proteins and phenylpropanoids, feeding-induced volatiles are emitted form many plant species (Van Poecke and Dicke 2004). Furthermore increasingly studies reveal that the wound-induced volatiles can transfer signal between plants (Engelberth et al. 2003; Karban et al. 2003). Methyl jasmonate (MeJA) emitted from infected plants by insects or pathogens (Kessler and Baldwin 2002) induces the accumulation of PIs in tomato leaves (Farmer and Ryan 1990). But in poplar plants, little evidence of interplant communication has been reported, and the role of MeJA is not completely understood.

In the present study, the activities of LOX and PAL in the leaves of poplar (*Populus simonii* × *P. pyramidalis* 'Opera 8277') cuttings after *Clostera anachoreta* larvae attack were measured, and the activities of LOX and PAL in the leaves of neighboring plants near to the wounded plants were also measured to investigate the interplant communication between poplar cuttings. Moreover, MeJA was used to treat intact plants to test its signaling role.

Materials and methods

Plant and animal materials

One-year-old poplar (*P. simonii* × *P. pyramidalis* 'Opera 8277') cuttings were cultured in pots (25-cm diameter, 25-cm height) containing nursery top soil under a 16-h photoperiod and 25/20°C day/night temperature regime in the greenhouse of Beijing Forestry University. They were watered daily and supplied with a full Hoagland nutrient solution every two weeks to avoid water and nutrient stress (Hu et al. 2004). The fourth instar larvae of *C. anachoreta* collected in Beijing Botanical Garden were placed in glass containers under 16-h photoperiod and at 23ºC. They were fed with fresh and intact leaves of poplar cuttings.

Insect herbivory and herbivore-induced volatiles exposure

The experiment was conducted in the greenhouse. Two individual cuttings were put in an air-tight glass chamber (100 cm×100 cm×100 cm), insuring that their leaves do not touch each other. Healthy and hungry larvae were released on the functional leaves (the sixth, seventh and eighth leaves from top) of one cutting (two larvae per leaf), and allowed to feed till about 25% of leaf area was wounded. The fourth and fifth leaves from the top of this cutting were used as the systemic leaves. Another intact cutting was exposed to herbivore-induced volatiles. The sixth, seventh and eighth leaves from top of this cutting were used as volatiles exposed leaves. There was an inlet on the chamber at the side of the wounded cutting and an outlet at the side of intact individual. A filter was fixed on the inlet to avoid microorganisms entering into the chamber. The air purified by activated carbon and GDX-101, was pumped into the chamber to draw the herbivore-induced volatiles to the neighboring intact cutting. At 0.5 h, 2 h, and 6 h after insect herbivory, the leaves, including wounded leaves and upper systemic leaves on the wounded cuttings and the volatiles exposed leaves on the neighboring intact plant, were harvested, and then dropped into liquid nitrogen. The leaves at the same positions of intact cuttings alone in another glass chamber at the same condition were used for control. Three replications were conducted for each treatment.

MeJA exposure

MeJA were purchased from Sigma/Aldrich. An intact poplar cutting was exposed to MeJA vapor in the same glass chamber with the final concentration of 1 μmol·L^{-1}. The poplar cutting was placed together with absorbent cotton on evaporation dishes, absorbing 1 mL of dilutions of MeJA in ethanol, or ethanol alone, as a control. The absorbent cotton was placed about 30 cm from the cuttings. After 6 h, the sixth, seventh and eighth leaves from top of the cuttings were collected and stored in liquid nitrogen. There were three replications for this treatment.

Enzyme activity determination

LOX was extracted and measured following the method of Axelrod et al. (1981) with some modifications. The leaves were ground to a fine powder with a mortar and pestle under liquid nitrogen, and then an ice-cold 0.1-mol·L^{-1} sodium phosphate buffer [pH 7.0, 0.1% (v/v) Triton X-100, and 1% (w/v) polyvinylpolypyrrolidone] was added. The homogenate was then centrifuged at 12000 g for 20 min at 4ºC. The supernatant was directly used as a crude extract for LOX activity assay. Linoleic acid substrate (10 mmol·L^{-1}) was prepared according to method of Axelrod et al. (1981) with some modifications. LOX activity was determined from the increase in optical density (OD). The reaction mixture consisted of 2.9 mL buffer, 50 μL linoleic acid substrate, and 10 μL enzyme extract. The results were expressed as $\triangle OD_{234}\cdot min^{-1}\cdot g^{-1}$Fw.

For the determination of activity PAL, the leaves were ground to a fine powder with a mortar and pestle under liquid nitrogen, and then an ice-cold borate buffer of 50 mmol·L^{-1}, pH 8.8, was added. The homogenate was then centrifuged at 12 000 g for 20 min at 4ºC. The supernatant was used as a crude extract for PAL activity assay. The reaction mixture contained 2-mL borate buffer, 0.8-mL L-phenylalanine, and 0.2-mL enzyme extract. First, the OD was measured at 290 nm, and after the reaction mixture was incubated at 30ºC for 30 min, the OD was measured again. The results were expressed as $\triangle OD_{290}\cdot min^{-1}\cdot g^{-1}$Fw.

Data analysis

Data were analyzed using one-way ANOVA for each individual date with treatments as the factor. One-way ANOVA was performed with Excel.

Results and discussion

In response to biotic and abiotic stresses, such as herbivore attack and mechanical damage, plants activate a series of defense mechanisms (Kessler and Baldwin 2002; Van Poecke and Dicke 2004). A direct defense, such as the synthesis of toxic secondary metabolites (Karban and Baldwin 1997) and PIs (Major and Constabel 2008), deters the feeding of insects. JA pathway plays a crucial role in the induction of defensive proteins (Farmer et al. 1992; Li et al. 2002a; Howe 2004; Li et al. 2004; Wang et al. 2008). LOX, as a key enzyme in JA pathway, showed high activity in the leaves of poplar cuttings after *C. anachoreta* larvae attack (Fig. 1A). It was observed that the insect herbivory induced a significant (p<0.05) enhancement of LOX activity (Fig. 1A). Moreover, from 0.5 h, the LOX activity exhibited a gradual increase, and the maximal activity occurred at 6 h. The LOX activity at 0.5 h and 6 h was almost 4.2 times and 7.9 times higher than that of the control, respectively. This elucidated that in response to insect herbivory, JA pathway was activated to contribute to the production of downstream defensive proteins in poplar cuttings. Phenylpropanoid pathway is an important pathway to produce phenylpropanoids and phenolics, as toxic secondary metabolites, to defend against environmental stresses (Dixon and Paiva 1995). PAL, a rate-limiting enzyme in phenylpropanoid pathway, also showed a significant (p<0.05) increase in activity in response to insect herbivory similar to LOX (Fig. 1B). At 6 h after insect herbivory, the maximal PAL activity increased by 7.2 times in wounded leaves. This indicated that this pathway was activated.

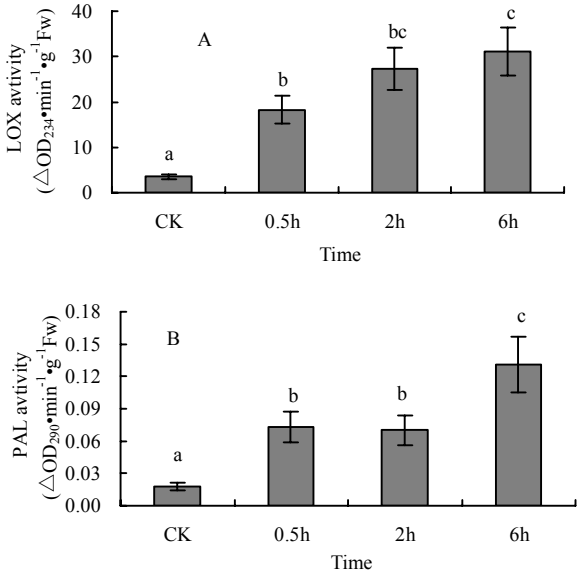

Fig. 1 Activities of LOX (A) and PAL (B) in leaves of *P. simonii* × *P. pyramidalis* 'Opera 8277' cuttings after insect herbivory. Values represent the mean and standard error of enzyme activities. Different letter indicates significant difference among time points (P<0.05).

The results demonstrated that JA and phenylpropanoid pathways producing defensive proteins and toxic secondary metabolites were triggered in *P. simonii* × *P. pyramidalis* 'Opera 8277' cuttings to defense against *C. anachoreta* larvae attack.

Increased activities of LOX and PAL were also found in upper systemic leaves (Fig. 2). A clear (p<0.05) increase in LOX activity was induced. The maximal LOX activity was about 5 times higher than that of control. And PAL activity in systemic leaves showed a 6.8-time increase. But the activities of LOX and PAL in systemic leaves were far less than that in wounded leaves at the same time point. For example, the maximal LOX activity in wounded leaves was 31.2 $\triangle OD_{234} \cdot min^{-1} \cdot g^{-1}$Fw, at 6 h after insect herbivory, while in upper systemic leaves, the value was 18.5 $\triangle OD_{234}$ $min^{-1} \cdot g^{-1}$ Fw. The results demonstrated that JA and phenylpropanoid pathways at the unwounded sites of poplar cuttings were activated, and defense system was also switched on providing strong support for the induced systemic resistance to insect herbivory in poplar plants. In previous studies, several signaling molecules, such as H_2O_2 (Orozco-Cárdenas et al. 2001) and JA (Li et al. 2002b) are thought to be the potential systemic signals in plants. But the systemic signals in poplar cuttings are little known, and need to be explored in the following study.

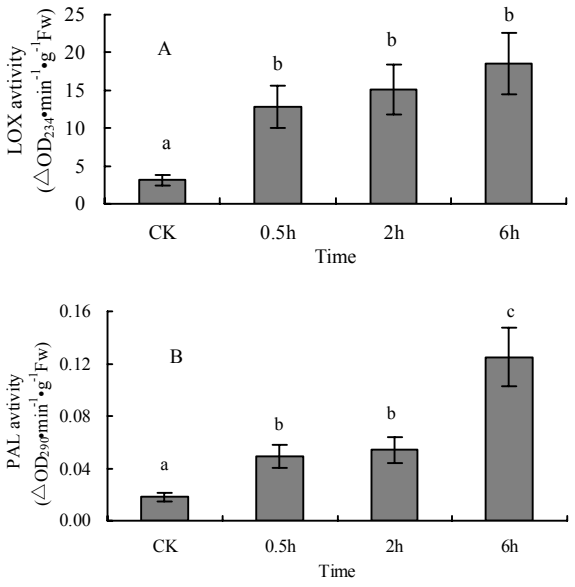

Fig. 2 Activities of LOX (A) and PAL (B) in upper systemic leaves of *P. simonii* × *P. pyramidalis* 'Opera 8277' cuttings after insect herbivory. Values represent the mean and standard error of enzyme activities. Different letter indicates significant difference among time points (P<0.05).

Baldwin and Schultz (1983) have reported that airborne signals synthesized in the tissue of damaged red ash plants stimulate biochemical changes in neighboring plants and suggested the existence of interplant communication. Increasing studies indicate that interplant communication probably is an import part of plant defense (Engelberth et al. 2003; Karban et al. 2003), and may play a key role in the population resistance of plants. In this study, the activities of LOX (Fig. 3A) and PAL (Fig. 3B) showed a significant (P<0.05) enhancement in the leaves of neighboring poplar cutting close to the herbivore-wounded poplar cutting,

compared to control, which also showed a gradual increase from 0.5 h to 6 h. The results provided evidence for communication between woody plants. The volatile signals emitted from wounded poplar cuttings transferred alarming information to neighboring intact plants, resulting in the stimulation of defense system in intact cuttings to deal with after insect herbivory.

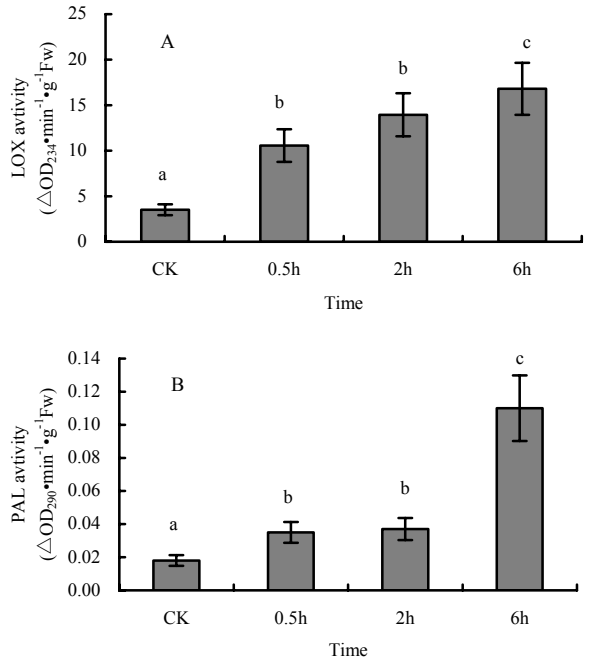

Fig. 3 Activities of LOX (A) and PAL (B) in the leaves of *P. simonii* × *P. pyramidalis* 'Opera 8277' cuttings exposed to herbivore-induced volatiles. Values represent the mean and standard error of enzyme activities. Different letter indicates significant difference among time points (P<0.05).

It has been reported that volatiles emitted from herbivore-wounded leaves of plants can serve as airborne signals to trigger defense response in intact plants (Engelberth et al. 2003). What are the airborne signals between plants becomes a hot research filed. MeJA is a well known airborne signal, and has been found to be emitted from infected plant by insects or pathogens (Schweizer et al. 1997; Rakwal and Komatsu 2000; Kessler and Baldwin 2002). It induces the accumulation of PIs in tomato leaves (Farmer and Ryan 1990). Treatment of barley seedlings with MeJA leads to a significant decrease in powdery mildew infection, as well as greatly increasing activity of defense-related enzymes in both treated leaves and systemic leaves (Walters et al. 2002). In our previous study, MeJA has been detected in the volatiles emitted from *P. simonii* × *P. pyramidalis* 'Opera 8277' cuttings after mechanical damage, despite a low content (data not shown). In this work, after exposure with 1-μmol·L^{-1} MeJA for 6 h, a significant (p<0.05) increase in activities of LOX (Fig. 4A) and PAL (Fig. 4B) was induced. The LOX activity induced by MeJA was 6.5 times higher than that of control, and a 1.1-fold increase was observed in PAL activity. Ethanol didn't cause a significant increase in these two enzymes. Hence, MeJA showed a potential signaling role between *P. simonii* × *P. pyramidalis* 'Opera 8277' plants. MeJA might diffuse into the cell cytoplasm where it would be hydrolyzed to JA by intracellular esterases. The acid may, in turn, be served as integral parts of general signal transduction systems that regulate defense genes in plants. But we thought that MeJA is impossibly the only airborne signal between *P. simonii* × *P. pyramidalis* 'Opera 8277' plants, and interplant communication may result from compound signal composed of different airborne signals.

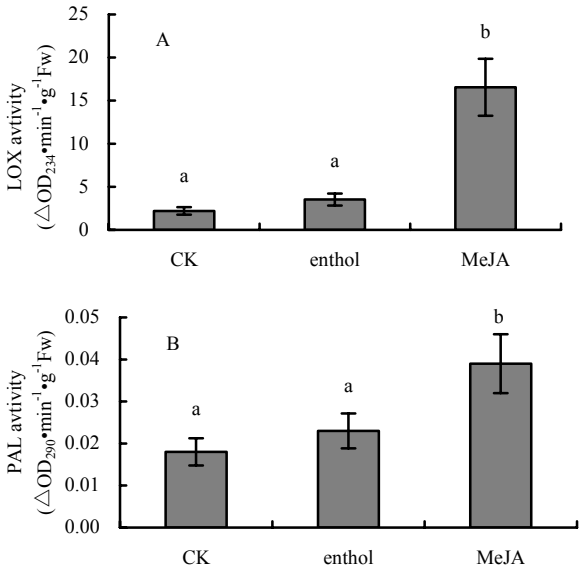

Fig. 4 Activities of LOX (A) and PAL (B) in the leaves of *P. simonii* × *P. pyramidalis* 'Opera 8277' cuttings exposed to MeJA. Values represent the mean and standard error of enzyme activities. Different letter indicates significant difference among treatments (P<0.05).

Conclusion

In this study, increased activities of LOX and PAL were found not only in the leaves wounded by *C. anachoreta* larvae but also in upper systemic leaves of *P. simonii* × *P. pyramidalis* 'Opera 8277' cuttings, indicating that JA and phenylpropanoid pathways were systemically activated to produce defensive proteins and toxic secondary metabolites. Furthermore, an increase in LOX and PAL activities in neighbouring intact poplar cuttings suggested that there was the interplant communication between poplar plants via the herbivore-induced volatiles. Also, MeJA were proved to an effective airborne signal to induce defense response in *P. simonii* × *P. pyramidalis* 'Opera 8277' cuttings. But the systemic signals and compound airborne signal in poplar plants are little known, which remains to be investigated.

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